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<b>(54) Title:</b> A METHOD FOR ASSAYING AND TREATING ALZHEIMER'S DISEASE  <b>(57) Abstract</b>  The present invention relates to a method of assaying for Alzheimer's disease in a human by determining the relative abundance of one or more forms of amyloid precursor protein (APP) or the enzyme responsible for said forms in circulatory fluid and to a method for treating the disease by modulating divalent cation, trivalent cation and/or heparin interaction with APP.		

## A METHOD FOR ASSAYING AND TREATING ALZHEIMER'S DISEASE

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The present invention relates to a method of assaying for Alzheimer's disease in a human by determining the relative abundance of one or more forms of amyloid precursor protein (APP) or the enzyme responsible for said forms in circulatory fluid and to a method for treating the disease by modulating  
10 divalent cation and/or heparin interaction with APP.

Alzheimer's disease is a progressive dementia characterised by the deposition of amyloid in the intracellular and extracellular compartments of the cerebral cortex (Davies *et al*, 1988). The extracellular deposits consist of a protein of  
15 4,000 relative molecular mass ( $M_r = 4K$ ) referred to as  $\beta A4$  (Kang *et al*, 1987). Molecular cloning and protein sequencing studies have shown that  $\beta A4$  comprises part of the membrane spanning and extracellular domains of the amyloid precursor protein (APP), which has features of an integral transmembrane cell surface receptor (Kang *et al*, 1987; Goldgaber *et al*, 1987).

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$\beta A4$  appears to result from abnormal cleavage of APP. Normal cleavage occurs at or near a lysine residue within the  $\beta A4$  sequence (Esch *et al*, 1990; Sisodia *et al*, 1990; Palmert *et al*, 1989). Cleavage at this site would prevent formation of the amyloidogenic  $\beta A4$  fragment. The enzyme which normally  
25 cleaves APP from the membrane surface is designated "APP secretase" although it has never been identified.

At least one form of APP has been shown to have neurotrophic activity, i.e. capable of promoting the survival or outgrowth of nerve processes. A disorder  
30 in APP processing may account for the loss of certain populations of neurons seen in Alzheimer's disease. There is now increasing evidence that APP is released from membranes, as part of its normal neurotrophic function, by

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cleavage at a site within the  $\beta$ A4 sequence (lysine 16) by APP secretase. As  $\beta$ A4 is produced in Alzheimer's disease, this indicates that a failure to cleave at this site might be the cause of Alzheimer's disease or at least contribute to the progression of the disease.

5

There is a need for an assay which is of predictive and diagnostic value in monitoring Alzheimer's disease and for any therapeutic interventions therein. In accordance with the present invention, it has now been discovered that processing of circulatory APP is altered in Alzheimer's disease thus providing a  
10 basis for an assay for the disease. Furthermore, from work leading up to the present assay, an improved means of treating Alzheimer's disease has been discovered based on modulating the interaction between divalent cations and/or heparin and APP.

15 Accordingly, one aspect of the present invention provides a method of assaying for Alzheimer's disease in a human said method comprising isolating a sample of circulatory fluid from said human, determining the amount of the 130 kDa form and/or 42 kDa form of APP and/or any derivatives of either form of APP in said fluid relative to a normal control wherein a relative increase in  
20 the 130 kDa form and/or its derivative and/or a relative decrease in the 42 kDa form and/or its derivative is indicative of the disease. By "assay" is meant screening or monitoring for the presence of, or a disposition to, Alzheimer's disease and/or to observe the effectiveness, or otherwise, of treatment of the disease following, for example, therapeutic intervention.

25

In accordance with the assay of the present invention, a sample of circulatory fluid is obtained from a test subject, generally after fasting since the prandial condition alters the level of APP in circulatory fluid, and the relative amounts of the 130 kDa and/or 42 kDa forms and/or their derivatives of APP  
30 determined. Fasting is generally for at least four hours but may be longer or shorter depending on the human to be tested. Fasting times may, for example, vary from 3 to 12 hours. Preferably, the circulatory fluid is blood plasma. A

number of means exist for determining the relative amounts and such determination may be quantitative or qualitative. Conveniently, a Western blot is performed using an antibody, and preferably a monoclonal antibody, which recognises the amino terminal portion of APP. Mouse monoclonal  
5 antibody (MAb) 22C11 (Boehringer Mannheim, Munich, Germany) is one such antibody. Following a Western blot, a reflective analysis can then be conducted by computing the integrated reflectance (i.e. the area under the curve) for the peak corresponding to the 130 kDa and/or 42 kDa forms of APP and/or their derivatives.

10

One skilled in the art will immediately recognise that other forms of quantitation or qualification are possible such as using HPLC and/or antibodies with different specificities to determine the relative levels of the different forms of APP. Such other methods are still within the scope of the present  
15 invention and include ELISA and competitive antibody assays. In any event, regardless of the means employed, it need only be determined that the 130 kDa form and/or its derivative of APP shows an increase and/or that the 42 kDa form and/or its derivative shows a decrease in their respective relative amounts to detect Alzheimer's disease and/or to monitor its progress.

20

The present invention also extends to genetic detection systems. These are of particular use in screening for people with a genetic predisposition to Alzheimer's disease. A genetic detection system, for example, may be based on a nucleic acid probe for the gene encoding the enzyme ("APPase")  
25 responsible for the alteration in circulatory APP.

According to this aspect of the present invention, there is provided a method of assaying for Alzheimer's disease in a human said method comprising isolating a sample of genetic material from said human, contacting said genetic  
30 material to an oligonucleotide capable of hybridising at or near the gene encoding APPase and ascertaining whether or not said APPase gene contains any nucleotide aberrations..

Preferably, the oligonucleotide is a probe and aberrations in the gene are determined by the extent of hybridisation to a region at or near said gene.

Alternatively, the oligonucleotide is a primer capable of directing amplification  
5 of DNA and aberrations are detected by sequencing of amplification products or measuring the profile of amplification products relative to a normal control.

In another embodiment, an antibody assay may be used to determine the amount of the enzyme. According to this embodiment there is provided a  
10 method for assaying for Alzheimer's disease in a human said method comprising isolating a sample of circulatory fluid from said human and contacting said fluid to a binding effective amount of an antibody specific to APPase and determining the amount of APPase in said fluid.

15 Preferably, the circulatory fluid is blood plasma.

Preferably, the antibody is a monoclonal antibody.

Conveniently, the binding of the antibody is determined by contacting the  
20 sample with a second antibody labelled with a reporter molecule and assaying a signed product by said reporter molecule.

In a specific embodiment the antibody is specific only for naturally occurring, active APPase.

25

By "derivative" means any single or multiple amino acid substitutions, deletions and/or additions to forms of APP occurring naturally or following direct or indirect therapeutic intervention. Accordingly, the present invention extends to monitoring the 130 kDa and/or 42 kDa forms or to any derivatives thereof  
30 which may possess higher or lower molecular weights. Hence, reference herein to the 130 kDa and 42 kDa forms of APP includes references to any derivatives thereof, where the relative amount of the derivative forms have th

same or similar predictive and diagnostic value in monitoring Alzheimer's disease as the 130 kDa and 42 kDa forms.

In a most preferred embodiment, the present invention provides a method for  
5 assaying for Alzheimer's disease in a patient, said method comprising isolating  
plasma from said patient and subjecting same to partial purification, identifying  
the 130 kDa and/or 42 kDa forms and/or their derivatives of APP in said  
partially purified plasma preparation by suitable means such as Western blot  
analysis using an antibody to the amino terminal portion of APP and then  
10 determining the amounts of said 130 kDa and/or 42 kDa forms and/or their  
derivatives relative to normal controls. By "partial purification" is meant that  
required to reduce interference by non-APP proteins and other molecules  
during the detection, such as by Western blot analysis. Conveniently, the  
partial purification is conducted by collecting the plasma in heparinised  
15 collection tubes, obtaining the plasma supernatant and subjecting same to  
heparin-Sepharose chromatography.

The present invention also relates to antibodies to the 130 kDa and/or 42 kDa  
forms and/or their derivatives of APP and to antibodies to APPase. The  
20 antibodies may be polyclonal or monoclonal antibodies or may be recombinant  
or chemically synthetic forms thereof. Such antibodies will be useful *inter alia*  
in the detection assay of the present invention. In this regard, these antibodies  
may be labelled with a reporter molecule such as an enzyme, radioisotope,  
chemiluminescent molecule, fluorescent molecule and the like. Alternatively, a  
25 labelled second antibody, specific to the first antibody, could be employed.  
The present invention, therefore, extends to the second antibody, whether or  
not the antibody is labelled. The said second antibody may be polyclonal  
monoclonal or synthetic as with the first antibody.

Yet a further aspect of the present invention provides a kit for assaying for Alzheimer's disease in a human said kit comprising in compartmental form a first compartment adapted to contain an antibody specific to the 130 kDa form  
5 and/or the 42 kDa form and/or derivatives thereof or to APPase; and optionally a second compartment adapted to contain a second antibody specific to said first antibody and labelled with a reporter molecule. The kit may also further comprise means for conducting a Western blot and/or some other quantitative or qualitative detection means. The kit may yet further comprise  
10 means for partially purifying the 130 kDa and/or 42 kDa forms and/or their derivatives of APP and/or APPase from the circulatory fluid such as plasma.

In yet another aspect of the present invention, there is provided a method for treating Alzheimer's disease in a patient in need thereof comprising subjecting  
15 said patient to means for modulating divalent cation and/or trivalent cation and/or heparin interaction with APP. Preferably, zinc interaction or heparin or any other moiety that binds to the heparin binding site with APP is modulated. This aspect of the present invention is predicated in part on the discovery that by manipulating the interaction between cations, preferably zinc,  
20 and APP, protease mediated digestion of APP (i.e. APPase activity) is altered.

An elevation in APP levels occurs in Alzheimer's disease. Elevations of APP mRNA are known to occur in the brains of sporadic Alzheimer's disease cases and are the most likely the pathogenetic event in the amyloidogenesis that  
25 invariably accompanies Down's Syndrome. Increases in APP levels can be induced in normal and Alzheimer's disease volunteers, in rats and in PC12 cultured cells by extracellular zinc loading. It is demonstrated herein that extracellular zinc modulates APP expression. This provides a basis for therapeutic intervention based on modulating divalent cation interaction with  
30 APP.



By modulating the levels of divalent cations or heparin or any other moiety which can bind the heparin binding sites on APP (residues 318-331 and around residues 98-105) or any other binding site on APP capable of binding these

5 moieties (such as additional zinc or heparin binding sites on APP), the range, type and/or extent of APP cleavage can be altered such that incorrect protease-mediated processing of APP can be reduced or inhibited. By "modulate" is meant the alteration of the availability of divalent cations and trivalent cations or heparin or any other moiety which can bind the heparin

10 binding sites on APP (residues 318-331 and around residues 98-105) or any other binding site on APP capable of binding these moieties (such as additional zinc or heparin binding sites on APP) to bind to APP prior to or simultaneously with APPase-mediated cleavage. It has been found that zinc ( $\text{Zn}^{2+}$ ) binds to APP at a specific and saturable binding site. The zinc

15 binding site on APP was identified by enzymatic digestion of purified APP695-fusion protein coupled to  $\text{Zn}^{2+}$ -chelating sepharose. The synthetic peptide representing about residues 181-200 of APP, situated between the cysteine rich and negatively charged domains of the protein, was shown to bind zinc in a specific and saturable manner. The intimate involvement between APP and

20 zinc is strongly suggestive of a role of zinc in APP processing: APP binds heparin (in a manner analogous to FGF). Heparin has been shown to protect APP from proteolytic digestion, as exemplified using the proteolytic enzyme trypsin. Heparin concentration as low as 100 nM cause a marked reduction in the rate and degree of brain APP degradation by trypsin. The brain contains a

25 number of heparin or heparin sulphate containing proteins and thus the interaction of heparin with APP may stabilise APP from proteolytic degradation in-vivo. It has also been found that zinc effects the kinetics of heparin binding to APP, and may increase APP affinity for heparin 5 to 10 fold. Surprisingly, at low zinc concentrations (above about 1  $\mu\text{M}$ ) the

30 protective effects of heparin are abolished. This finding indicates that aberrant zinc levels in-vivo, in the brain intracellular and/or extracellular milieu, may promote aberrant APP proteolytic processing giving rise to the amyloid

protein, and subsequently Alzheimer's disease and other disorders associated with amyloid deposition in the brain.

5 The mechanisms behind zinc abolishing the protective effects of heparin are uncertain.

Studies conducted in Alzheimer's disease patients demonstrate that administration of zinc (such as elemental zinc in the form of sulphate) at a moderate oral dosage such as 50 to 100 mg of grams per day over several days  
10 (in keeping with conventional zinc supplements obtainable from pharmacies) leads to rapid deterioration of neural functioning as demonstrated by a severe loss of cognitive function with mini-mental state examination (Folstein et al., 1975) scores deteriorating from moderately demented levels to unrecordable. Eye movement abnormalities and general levels of self care worsened over the  
15 period of supplementation. In contrast, healthy volunteers showed no ill effects from zinc supplementation.

The results obtained from Alzheimer's patients is consistent with a neurotoxic response to zinc metabolism in the brain in Alzheimer's disease.  
20

In one aspect of this invention Alzheimer's disease and other neurological disorders are treated, ameliorated and/or prevented by administering to a patient in need of such treatment a therapeutically effective amount of a zinc binding agent which agent is capable of binding a divalent or trivalent cation  
25 and thereby modulating its interaction with APP. More particularly, the cation is a divalent cation and even more particularly is zinc and the binding agent is a zinc binding agent. This modulation may comprise reducing the bioavailability of zinc due to the formation of complexes with zinc thus reducing free zinc. Zinc binding may, for example, take place in the  
30 gastrointestinal tract, in the blood stream and/or in the brain, such as at an extracellular and/or intracellular level.

Any pharmaceutically acceptable zinc binding agent may be employed in this invention. Particularly preferred are binding agents which are capable of crossing the blood/brain barrier and thus modulate free zinc concentrations within the brain at an extracellular and/or intracellular level in order to restore aberrant zinc levels in the brain thus protecting against improper APP processing which may give rise the amyloid protein. Examples of zinc binding agents (such as chemical chelators) include phytic acid and derivatives thereof (such as phytate), desferrioxamine, sodium citrate, ethylene diamine tetra acetic acid (EDTA), and zinc specific chelating agents based on heterocyclic pyridones such as 1,2-diethyl-3-hydroxypyridin-4-one (CP94) and 1-hydroxyethyl-3-hydroxy-2-methylpyridin-4-one (CP40) (Hilder et al., 1990), which agents may be capable of crossing cell membranes (CP94) or incapable of permeating cells (CP40).

Zinc interaction with APP may be modulated by diet, by administering to patients a low zinc diet, or removing dietary sources of zinc. Zinc is enriched in numerous foodstuffs. Prominent amongst these are oysters, crab, beef, liver and other seafood and animal products (Stanton, 1992). The bioavailability of zinc is inhibited by unprocessed wheat bran, high alcohol and various proteins. Avoidance of animal products combined with a diet containing unprocessed fibre, such as wheat bran, may reduce the bioavailability of zinc and hence reduce the contribution of zinc to neurotoxicity. Thus, in accordance with this aspect there is provided a method for treating/ameliorating or preventing Alzheimer's disease which comprises administering to a subject in need of such treatment a diet low in free zinc.

The term "modulate" extends to the use of pharmacological agents which disrupt zinc transport mechanisms across cell membranes. Like other metal ions, such as iron and calcium, zinc is transported into cells via a zinc transport system (currently poorly characterised) which may involve one or more proteins and/or lipids and/or carbohydrates which regulates zinc flow across

membranes. Pharmacological agents which disrupt one or more components of the zinc transport system may be used to block zinc uptake from the intestines as well as zinc transport into and out of cells in the brain. Accordingly, there is provided in this aspect a method for modulating zinc

5 interaction with APP which comprises administering to a subject a pharmacological agent capable of blocking one or more components of the zinc transport system so as to reduce zinc uptake. Using such agents it may be possible to correct the maldistribution of zinc in extracellular and intracellular compartments in Alzheimer's disease. The term "modulate" further extends to

10 means for affecting the interaction of the cations to APP. Such means include, for example, changes in pH. "Modulate" also extends to altering zinc metabolism with agents such as iron supplements which, for example, suppress zinc absorption from the gut and promote zinc elimination, or by blocking the cation binding site on APP or on the cation responsive promoter region of the

15 APP gene, for example with cupric ions.

Administration of zinc binding compounds and pharmacological agents capable of disrupting the zinc transport system may be by parenteral or oral administration although all other known modes of administration are

20 contemplated by the present invention.

Sustained high concentrations of extracellular zinc (greater than 200  $\mu\text{M}$ ) are known to be neurotoxic. Zinc concentrations in hippocampal synapses briefly reach 300  $\mu\text{M}$  during synaptic transmission. The disruption of extracellular

25 zinc metabolism may be an important step in the neurotoxic mechanism that accompanies amyloid deposition in Alzheimer's disease. The findings disclosed herein support the proposition that APP is important in the regulation of neuronal zinc compartmentalisation. An abnormality of APP metabolism may consequently cause an abnormality of zinc metabolism. A strategy to remedy

30 this abnormality in Alzheimer's disease would involve the restoration of normal APP metabolism, for example by reversing the abnormal APP protease resistivity that occurs in Alzheimer's disease, or by treating the Alzheimer's

disease patient with supplements of normal APP.

It is to be understood that aspects of this invention involving modulation of zinc levels run counter to previously proposed therapies which suggested

5 Alzheimer's disease may be associated with zinc deficiencies and thus proposed administering zinc to patients suffering from Alzheimer's disease. As described herein it has been found that administration of zinc to Alzheimer's disease patients worsens the disease, this being evaluated by standard neurological testing.

10

It has surprisingly been found that Alzheimer's disease may be detected by administering to a subject a challenge of zinc, and thereafter testing neurological function according to one or more standard tests as are well known in the art. When compared to non-Alzheimer's normal controls, a  
15 person suffering from Alzheimer's disease shows a decrease in cognitive abilities as well as a decrease in other standard neurological function tests. One convenient test is an assessment of eye movement to visual stimuli which is reduced markedly in Alzheimer's disease patients on zinc challenge compared to normal controls. The amount of zinc administered to a subject in  
20 a challenge test would generally comprise from 50 to 500 mg or more. The precise amount of zinc administered in a test is not crucial and would generally be based on minimising side effects to zinc administration in Alzheimer's patients.

25 Thus, in a further aspect of this invention there is provided a method for detecting Alzheimer's disease which comprises administering to a subject a challenge of zinc and thereafter assessing neurological function, where a decrease in neurological function compared relative to normal controls is indicative of Alzheimer's disease.

30

The zinc challenge may be administered to a patient in various ways, such as orally, intravenously, intramuscularly, transdermally, rectally, intranasally and the like. Oral administration is preferred. As mentioned above, the amount of zinc administered to a patient in a challenge test is not critical as long as the amount administered is capable of evoking a response in Alzheimer's patients without precipitating severe disease symptoms.

The present invention is further described by reference to the following non-limiting figures and examples.

In the Figures:

**FIGURE 1** is a photographic representation showing immunoblots comparing Alzheimer's disease (B) and age matched control plasma APP (A). Plasma heparin-Sepharose eluates (65 µg) were analysed by 8.5% (w/v) SDS polyacrylamide gel electrophoresis and immunoblotting with MAb 22C11 which recognises an amino-terminal epitope (see Example 1). The relative molecular mass of standard protein markers (Rainbow Standards, Amersham, UK) are shown on the left. APP immunoreactive bands of 130, 110 (a doublet), 65 and 42 kDa are indicated by arrows to the right. Only the relative abundances of the 130 and 42 kDa APP forms, as in the sample illustrated, could visibly discriminate between Alzheimer's disease compared to (A) non-demented elderly controls and (B) normal young control populations.

**FIGURE 2** is a photographic representation showing a comparison of APP proteolytic activity from Alzheimer's disease (B) and control plasma (A). Heparin-Sepharose purified APP from plasma of Alzheimer's disease and control cases were incubated for 2 hours at 37 °C in saline buffer in the presence or absence of 20 µM  $Zn^{2+}$ . Samples (65 µg of protein) of each incubation were analysed by electrophoresis on 8.5% (w/v) polyacrylamide gels and immunoblotted with 22C11. The relative molecular mass of standard protein markers (Rainbow Standards, Amersham, UK) are shown on the left.

APP immunoreactive bands of 130, 110, 65 and 42 kDa are indicated by arrows on the right. The figure shows samples representative of six Alzheimer's disease cases and six normal young adult controls.

5 **FIGURE 3** is a photographic representation of a Western blot of platelets and plasma from gray platelet syndrome compared to control. An aliquot of 65µg of plasma purified by heparin-Sepharose chromatography and 50µg of washed whole platelets from a patient with gray platelet syndromes (GPS) were compared to similar preparations obtained from a young adult control. The  
10 samples were analysed by 10% (w/v) polyacrylamide mini-gel electrophoresis and immunoblotted with 22C11. The relative molecular mass of standard protein markers (Rainbow Standards, Amersham, UK) are shown on the left. The positions of major immunoreactive bands in normal platelets (130 and 110 kDa) and a minor band (65 kDa) are indicated by arrows on the right.

15

**FIGURE 4** is a graphical representation showing a time course analysis of <sup>65</sup>Zn binding to human brain 130/110 kDa APP. Amyloid protein precursor (APP) was purified according to the method in Moir et al. (1992). The preparation of purified 130 and 110 kDa APP was derived from human brain  
20 membrane extracts and contained the full-length precursor with carboxyl terminus intact but lacked the 17 residue signal peptide. The 130 and 110 kDa proteins were apparent in equal ratios on silver staining following polyacrylamide gel electrophoresis and were the only bands visible. The identity of these two proteins was confirmed by Western blots with monoclonal  
25 antibody 22C11 (which recognises an amino terminal epitope on APP), and by amino terminal sequencing. The protein concentration of the APP preparation was determined by amino acid analysis. Aliquots of APP (90 ng, ≈1.1 pmole, assuming the average amino acid formula weight of APP is ≈80 kDa) were incubated at 20 °C for the times indicated. Incubations (50 µl) were  
30 performed in 150mM NaCl, 50mM Tris-HCl, pH 7.4 in the presence of 10<sup>6</sup> cpm of <sup>65</sup>Zn (175 nM). The incubation solution was then applied to a 1.1 ml bed volume Sephadex G25 (Pharmacia, Uppsala, Sweden) column pre-

equilibrated with 150mM NaCl, 50mM Tris-HCl, pH 7.4 and allowed to settle before being desalted with 645  $\mu$ l of the Equilibration buffer. Previous analysis of the desalting properties of the column with mixtures of Dextran Blue and potassium dichromate solution indicated that >95% of the protein in the incubation mixture would be desalted into this volume with no detectable free salt present. The desalted protein was collected directly into counting tubes containing 10 ml aqueous counting scintillant. Less than two minutes was required to complete the desalting. The amount of  $^{65}\text{Zn}$  bound to desalted APP was determined by counting the collected sample in a beta-counter set to the broadest channel. Counting was determined to be 51% efficient. The values shown are means  $\pm$  SD of  $n \geq 3$  readings. These data indicate that rapid binding of Zn to APP occurs ( $\approx 30\%$  of  $B_{\text{max}}$  at 5 minutes) and reaches a maximum at 30 mins.

FIGURE 5 is a graphical representation showing a competition analysis of  $^{65}\text{Zn}$  binding to human brain 130/110 kDa APP. Aliquots of APP were incubated for 30 minutes with  $^{65}\text{Zn}$  as in Figure 4. The binding of the labelled  $\text{Zn}^{2+}$  to APP was subjected to competition with unlabelled  $\text{Zn}^{2+}$  in the form of the chloride salt. Data shown indicate the competition curves generated at pH 6.4 and 7.4. The binding of  $\text{Zn}^{2+}$  to APP deteriorated at the lower pH ( $\approx 45\%$  of  $B_{\text{max}}$ , pH 7.4). The values are means  $\pm$  SEM of  $n \geq 3$  readings. The curves illustrated are typical of three experiments.

FIGURE 6 is a graphical representation depicting a Scatchard analysis of  $^{65}\text{Zn}$  binding to human brain 130/110 kDa APP. Derived from the data points in Figure 5, Scatchard analysis reveals that the dissociation constant ( $K_D$ ) for the  $\text{Zn}^{2+}$  binding site on APP is 764 nM at pH 7.4 and 2.08  $\mu\text{M}$  at pH 6.4. One APP molecule binds one zinc ion.



FIGURE 7 is a graphical analysis showing a specificity study of the  $Zn^{2+}$  binding site on APP.

- (a) APP incubation with  $^{65}Zn$  and competing unlabelled  $Zn^{2+}$  was performed under the conditions detailed in Figure 5. In addition, the effect of competition for  $Zn^{2+}$  binding by  $Ca^{2+}$  and  $Mg^{2+}$  is shown. The values are means  $\pm$  SEM of  $n \geq 3$  readings. The competition curves for  $Ca^{2+}$  and  $Mg^{2+}$  are shifted greater than two log units to the right of the  $Zn^{2+}$  competition curve, indicating that the binding site is more specific for  $Zn^{2+}$  at physiological concentrations.
- (b) Comparison was made of the ability of other metal ions to compete with  $^{65}Zn$  for binding to APP. APP incubations with  $^{65}Zn$  and competing unlabelled metal ions (at 20  $\mu M$ ) were performed under the conditions detailed in Figure 5. The values are means  $\pm$  SEM of  $n \geq 3$  readings.  $Zn^{2+}$  could compete >97% of the label off the APP at 20  $\mu M$ .  $Co^{2+}$  was the next most competitive metal ion, competing  $\approx 70\%$  of the label off the APP at the same concentration.

- FIGURE 8 is a photographic representation showing an analysis of immunoreactive APP in plasma by western blot. APP immunoreactive proteins in heparin-Sepharose eluates from plasma were analysed by 8.5% SDS polyacrylamide gel electrophoresis and western blotting with mAb 22C11. Lane 1: Heparin-Sepharose eluate of plasma (65  $\mu g$  of protein). Lane 2: Heparin-Sepharose eluate immunoprecipitated by antiserum 90/3 (raised against full-length human brain APP). Lane 3: Heparin-Sepharose eluate immunoprecipitated by the prebleed to antiserum 90/3. Lane 4: Heparin-Sepharose eluate immunoprecipitated by anti-Fd-APP (raised against APP fusion protein). The relative molecular masses of standard protein markers (Rainbow Standards, Amersham, UK) are shown on the left. APP immunoreactive bands previously reported<sup>15</sup> of 130, 110 (a doublet), 65 and 42 kDa, are indicated by arrows on the right.

FIGURE 9 is a graphical representation of scattergrams of reflectance analysis of immunoblots comparing AD and control plasma APP. The distribution of plasma APP immunoreactivity was analysed by reflectance as detailed in Table 1. Solid lines indicate the means for each subgroup. A significant difference in the levels of 130 and 42 kDa species of APP was seen between AD and pooled control groups. Therefore, further analysis was performed on the differences in the concentrations of these species between these groups. (a) Proportions of 130 kDa APP in the AD group and individual control groups. The means proportion of 130 kDa APP species ranged approximately 50% to 80% ( $p \leq 0.05$ ; Scheffé, 1959) greater than the AD group compared to each control group. The dotted line indicates a suggested threshold for a biochemical characterization of AD providing 91% specificity and 78% sensitivity. (b) Proportions of 42 kDa APP concentrations in the AD group compared to the individual control groups. The mean proportion of the 42 kDa APP species ranged approximately 30% to 40% ( $p \leq 0.05$ ; Scheffé, 1959) lower in the AD group compared to the young adult and the age-matched control groups. The dotted line indicates a threshold for a biochemical characterization of AD providing 85% specificity and 50% sensitivity.

20

### EXAMPLE 1

#### MATERIALS AND METHODS

##### Materials:

All reagents were analytical grade except for the Tris-HCl which was electrophoresis grade (BioRad) to avoid contamination with traces of zinc.  $^{65}\text{Zn}$  was purchased from Amersham (U.K.).

##### Case selection:

Alzheimer's disease cases met NINCDS/Alzheimer's disease RDA clinical criteria (McKann *et al*, 1984) and had Mini-mental state (Folstein *et al*, 1975) scores of less than 17. Age-matched controls each underwent a Mini-mental state examination and were excluded if they scored less than 28. The different neurological diagnoses used as non-demented neurological disease controls

(n=6) were epilepsy, demyelinating disease, hydrocephalus and three cases of cerebrovascular disease. All volunteers were in stable health, not suffering any acute illness, at the time of the study.

5 Partial purification of APP from plasma:

Blood (20-40ml) was drawn from fasting individuals within a 21-gauge needle into heparinized collection tubes and centrifuged at 2500 g for 15 minutes.

The plasma supernatant fraction was separated from the blood cell pellet and centrifuged at 19,000g for 25 minutes at 4 °C (J2-21 centrifuge, Beckman,

10 USA) to remove any debris.

To detect APP by Western blotting, APP was partially purified from plasma by heparin-Sepharose chromatography. Plasma (2.5ml) was loaded onto a 0.25 ml bed volume heparin-Sepharose (Pharmacia, Uppsala, Sweden) column (8 mm x  
15 5 mm) pre-equilibrated with buffer 1 (175 mM NaCl, 50 mM Tris-HCl, pH 7.4) at 4 °C. The column was then washed with 3.25 ml buffer 1 and the APP eluted with 750 µl elution buffer (550 mM NaCl, 50 mM Tris-HCl, pH 7.4). Protein concentration was determined with BCA using bovine serum albumin standards (Pierce, Rockford, IL, USA) according to the method of Smith et al,  
20 (1985).

Washed platelets were prepared according to the method of Bush et al. (1990). Platelets ( $1.2 \times 10^7$ ) were solubilized in 30 µl of sample buffer (100 mM Tris-HCl, 2% (w/v) sodium dodecyl sulfate (SDS), 0.01% (w/v) Bromophenol blue,  
25 5% (v/v) β-mercaptoethanol (pH 6.8) and boiled for 10 minutes before Western blotting from polyacrylamide gels.

Plasma zinc assay:

Zn<sup>2+</sup> assays were performed by atomic absorption spectrophotometry  
30 according to the method in Davies et al. (1968).

#### Western blotting:

Western blotting procedures were as described by Bush *et al.* (1990). Blots were probed with a mouse monoclonal antibody (mAb) 22C11 (Boehringer Mannheim, Munich, Germany), which recognises an epitope on the amino-terminus of APP (15), diluted 1:60,000 in blocking buffer. Plasma samples in this series of experiments were separated on 8.5% (w/v) polyacrylamide gels unless otherwise stated. Using these conditions, the 110 kDa APP immunoreactive band resolved into a doublet. However, for the purpose of the present analysis the sum of the signals generated by the doublet are regarded as belonging to the one 110 kDa region.

#### Reflectance analysis of blots:

Reflectance analysis of blots was carried out by video-capture with a Videk Megaplus camera (Kodak, Canandaigua, NY, USA) operated by PixelTools v1.1 (Perceptics, 1990). Quantitation was then performed with Image v1.29 software (W. Rasband, National Institutes of Health Research Services Branch, NIMH) which facilitated precise alignments of the individual blot lanes with the reflectance profile and the setting of exclusion limits of individual peaks in the four regions of interest at 130, 110, 65 and 42 kDa. The integrated reflectance (area under the curve) was thus computed for each of the four peaks in every sample. The values obtained were linear with concentration for each region over a range of plasma heparin-Sepharose eluate doses (20-90 µg of protein).

25

To compare the relative amounts of the four APP derivatives, the relative percentage of band signal to total lane signal was determined in each plasma sample and then averaged to give the values presented in Table 1.

Independent samples Student's t-tests between pooled controls and Alzheimer's disease groups were performed for the four immunoreactive bands at a significance level of  $p=0.0125$  (0.05 divided by the number of comparisons).

Where those comparisons were significantly different, the test of simple effects

(Weidemann *et al*, 1989) followed by Scheffe (1959) *post hoc* comparisons between all pairs of diagnostic groups (Alzheimer's disease, other neurological disease controls, normal young adult controls and non-demented age-matched controls) were then performed.

5

#### Assay of APP-degrading protease:

Aliquots (500  $\mu$ l) of eluates from each heparin-Sepharose column were desalted using a 1.7 ml Sephadex G25 (Pharmacia, Uppsala, Sweden) column (8 mm x 34 mm) equilibrated with 175 mM NaCl, 50 mM Tris-HCl, 1 mM  
10  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , pH 7.4, in the presence or absence of 20  $\mu$ M  $\text{ZnCl}_2$  at 4 °C, and the protein concentration adjusted to 0.80 mg/ml with the same buffer. The samples were then incubated at 37 ° for 2 hours, then an aliquot (81.3  $\mu$ l) was removed, and the protein in each aliquot was precipitated with chloroform/methanol (Wessel and Flugge, 1984), boiled in SDS sample buffer  
15 and analysed by Western blotting using MAb 22C11.

The effects of inhibitors of various classes of proteases were assayed by adding them to these incubation mixtures and observing their influence upon the degradation of 130 kDa APP. A sample (500  $\mu$ l) of a heparin-Sepharose  
20 eluate from the plasma of a normal young adult control was desalted into  $\text{Zn}^{2+}$ -buffer (175mM NaCl, 50 mM Tris-HCl, 1mM  $\text{CaCl}_2$ , 1mM  $\text{MgCl}_2$ , 20 $\mu$ M  $\text{ZnCl}_2$ , pH 7.4). Aliquots (containing 65 $\mu$ g protein) were diluted to 0.80 mg/ml with the same buffer containing protease inhibitor, then incubated for 2 hours at 37 °C. Protein was precipitated in each sample by the addition of  
25 chloroform/methanol (as above) and immunoblotted. The final concentrations of inhibitors in the incubation mixtures were EDTA (1 mM), diisopropyl fluorophosphate (DFP) (1 mM), aprotinin (10  $\mu$ g/ml), N-ethylmaleimide (NEM) (1 mM), pepstatin A (10  $\mu$ g/ml),  $\alpha$ 1-antichymotrypsin (0.4 mg/ml) and soya bean trypsin inhibitor (SBTI) (1 mg/ml). The effects of  $\text{Al}_3\text{Cl}$  (20  $\mu$ M)  
30 and heparin (20 U/ml) were also measured in this system.

**Brain APP preparation:**

Amyloid protein precursor (APP) was purified according to the method in Moir et al. (1992). The preparations of purified 130 and 110 kDa APP were  
5 derived from human brain membrane extracts and contained the full-length APP with carboxyl terminus intact, but lacked the 17 residue signal peptide. The 130 and 110 kDa proteins were apparently in equal ratios on silver staining following polyacrylamide gel electrophoresis, and were the only bands visible. The identity of these two proteins was confirmed by Western blots  
10 with monoclonal antibody (mAb) 22C11 (which recognises an amino terminal epitope on APP [Weidemann et al., 1989]), and by amino terminal sequencing. The protein concentration of the APP preparations was determined by amino acid analysis.

**15 Human serum preparation:**

40-60 ml of whole blood was allowed to clot for 3 hours in plain tubes at 20 °C. The clotted blood was then incubated for 16 hours at 4 °C, whereupon it was centrifuged at 1500 g for 15 minutes and the supernatant removed. The serum was further centrifuged at 1500 g for 15 minutes and the cell-free  
20 supernatant removed.

 **$^{65}\text{Zn}^{2+}$  binding to APP:**

Binding analysis was performed as follows: Aliquots of APP (90 ng,  $\approx 1.1$  pmole, assuming the average amino acid formula mass of APP is  $\approx 80$  kDa)  
25 were incubated at 20 °C. Incubations (50  $\mu$ l) were performed in 150mM NaCl, 50mM Tris-HCl, pH 7.4 in the presence of  $10^6$  CPM of  $^{65}\text{Zn}$  (175 nM). The incubation solution was then applied to a 1.1 ml bed volume Sephadex G25 (Pharmacia, Uppsala, Sweden) column pre-equilibrated with 150 mM NaCl, 50 mM Tris-HCl, pH 7.4 and allowed to settle before being desalted with 645  $\mu$ l  
30 of the equilibration buffer. Previous analysis of the desalting properties of the column with mixtures of bovine serum albumin and potassium dichromate solution indicated that >95% of the protein in the incubation mixture would

be desalted into this volume with no detectable free salt present. The desalted protein was collected directly into counting tubes containing 10 ml aqueous counting scintillant (ACSII, Amersham). Less than two minutes was required to complete the desalting. The amount of  $^{65}\text{Zn}$  bound to desalted APP was  
5 determined by counting the collected sample in a beta-counter set to the broadest channel. Counting was determined to be 51% efficient. The binding of the labelled  $\text{Zn}^{2+}$  to APP was subjected to competition with unlabelled  $\text{Zn}^{2+}$  and other metal chloride salts.

10 **Characterisation of the putative zinc-binding domain of APP:**

16  $\mu\text{g}$  of the synthesized peptide  
GVEFVCCPLAEESDNVDSADAEEDDSVWWGGAD, representing  
residues 181-214 of APP<sub>695</sub> (or residues 181-200), or 16  $\mu\text{g}$  of control peptide,  
was dissolved in 200  $\mu\text{l}$  of blocking buffer (50 mM Tris-HCl, 1 mM  $\text{MnCl}_2$  10  
15 mM  $\beta$ -mercaptoethanol, 20% methanol, pH 7.4) and dot-blotted onto PVDF  
(Immobilon-P, Millipore, Bedford, MA) which had been pre-wetted with  
methanol. The blot was then incubated for 30 mins at 20°C with 100,000  
CPM of  $^{65}\text{Zn}^{2+}$  in blocking buffer, in the presence or absence of various  
concentrations of competing unlabeled  $\text{Zn}^{2+}$  or other divalent cations. After  
20 incubation the dot-blot was washed three times with 200  $\mu\text{l}$  of blocking buffer  
without  $\text{MnCl}_2$ , the dot was excised and placed in 10 ml of scintillant and  
assayed by  $\beta$ -counting.

**Iodination of APP:**

25 100 kDa human brain full-length APP was iodinated by the Chloramine-T  
method. Iodinated APP ( $^{125}\text{I}$ -APP) was then separated from labelling  
reagents by Sephacryl G25 chromatography.

APP binding to heparin-Sepharose:

$^{125}\text{I}$ -APP (0.37 pmol, 40,000 CPM) was loaded in 250  $\mu\text{l}$  of buffer 1 (50 mM Tris-HCl, 0.1% BSA, pH 7.4  $\pm$  1 mM EDTA or 25-150  $\mu\text{M}$   $\text{ZnCl}_2$ ) onto a 0.25  
5 ml bed volume heparin-Sepharose column (8 mm x 5 mm) pre-equilibrated with buffer 1 at 20°C. The column was then washed with 6 ml buffer 1 and the APP eluted with aliquots of high-NaCl buffers. Gradient elutions were performed by eluting in 50 mM increments (700  $\mu\text{l}$ ) of the NaCl concentration from 0 to 1200 mM followed by a pulse of 2000 mM NaCl (in 50 mM Tris-  
10 HCl, 0.1% BSA, pH 7.4  $\pm$  1 mM EDTA or 50  $\mu\text{M}$   $\text{ZnCl}_2$ ). Pulse elution of APP bound to heparin-Sepharose employed 700  $\mu\text{l}$  of buffer 2 (50 mM NaCl, 50 mM Tris-HCl, 0.1% BSA, pH 7.4  $\pm$  1 mM EDTA or 25-150  $\mu\text{M}$   $\text{ZnCl}_2$ ) followed by 700  $\mu\text{l}$  of buffer 3 (100 mM CaCl<sub>2</sub>, 50 mM Tris-HCl, 0.1% BSA, pH 7.4  $\pm$  1 mM EDTA or 25-150  $\mu\text{M}$   $\text{ZnCl}_2$ ), followed by 700  $\mu\text{l}$  of buffer 4 (500  
15 mM NaCl, 50 mM Tris-HCl, 0.1% BSA, pH 7.4  $\pm$  1 mM EDTA or 50-150  $\mu\text{M}$   $\text{ZnCl}_2$ ), followed by 2.5 ml of regenerating buffer (2000 mM NaCl).

APP binding to heparin-Sepharose was also studied using partially purified APP preparations derived from human brain membranes (according to the  
20 method of Moir et al, 1992) or from human serum. Washed brain membrane extract or human serum was adjusted to a NaCl concentration of 350 mM and applied to a Q-Sepharose column (5 cm x 5 cm), washed with 350 mM NaCl, 50 mM Tris-HCl, pH 7.4 (250 ml [x 2 for brain membrane extract]) and eluted with 1 M NaCl 50 mM Tris-HCl, pH 7.4 (200 ml at 5 ml/min). This  
25 procedure causes an 80-fold enrichment of APP in the peak protein fractions, which are then pooled (Moir et al, 1992). The eluate (20 ml) was desalted into 25 mM Tris, pH 7.4 (30 ml) using a G25 Sephadex column (2.6 cm x 40 cm). 15 ml of the product was adjusted to 50  $\mu\text{M}$   $\text{Zn}^{2+}$  with 2 mM  $\text{ZnCl}_2$ , 50 mM Tris-HCl, pH 7.4 and the other 15 ml was adjusted to the same volume  
30 with 50 mM Tris-HCl, pH 7.4. 10 ml of the desalted Q-Sepharose eluate, containing 6.7 mg protein, was applied to heparin-Sepharose (1.6 cm x 5 cm) at 0.35 ml/min, washed with 100 ml of loading buffer, and eluted by a 0 to 1500



mM NaCl gradient ( $\pm 50 \mu\text{M Zn}^{2+}$ ) in 52.5 ml at a flow rate of 0.35 ml/min. Fractions (1 ml) representing 20 mM NaCl increments were collected and 30  $\mu\text{l}$  samples of each fraction were assayed for APP by Western blot with mAb 22C11 according to the method in Bush et al. (1990), modified by using PVDF membranes and by reducing the blocking time to 1 hour. The immunoreactive staining of the 130 kDa APP species seen on the blots was quantified by computer-assisted image capture reflectance densitometry (Bush et al., 1992). The 130 kDa reflectance signals on the blots were expressed as the ratio of the signal generated in one sample lane related to the 130 kDa APP reflectance reading of a sample of starting material present on every individual filter as an internal standard.

## EXAMPLE 2

### 15 Abnormal profile of plasma APP in Alzheimer's disease:

A monoclonal antibody (22C11), which recognises the amino-terminus of APP (Weidemann *et al*, 1989) identified four major immunoreactive bands of APP (130, 110, 65 and 42 kDa) in western blots of human plasma (Bush *et al*, 1990; Buch *et al*, 1991). The relative abundance of these bands was surveyed in Alzheimer's disease and controls. The 130 kDa plasma APP band was increased and the 42 kDa plasma APP band was decreased in Alzheimer's disease cases compared to controls. The controls consisted of groups of non-demented age-matched persons (Figure 1A), normal young adults (Figure 1B) and other neurological disease patients. There was no consistent difference in the levels of the 110 and 65 kDa bands between Alzheimer's disease and controls. The total amount of APP immunoreactivity did not differ between Alzheimer's disease and control as the ratio of the total immunoreactivity of Alzheimer's disease: controls was  $1.02 \pm 0.22$  (mean  $\pm$  SD).

30 Quantitation of these findings by image capture analysis (Table 1) showed that the 130 kDa and 42 kDa bands in AD were significantly (two-tailed,  $p < 0.001$ ) increased and decreased, respectively, compared to pooled controls

(averaged data from other neurological disease controls, normal young adult controls and non-demented age-matched controls). In Alzheimer's disease there was a 60% increase in the proportion of the 130 kDa form and a concomitant 35% decrease in the 42 kDa form. The immunoreactivity pattern was more evenly distributed in the pooled control group where the most concentrated APP immunoreactivity was in the 42 kDa region. This trend was maintained throughout the comparisons made of the Alzheimer's disease cases with the three control subgroups where *post hoc* analysis confirm the significance of the difference between the Alzheimer's disease and each control group in the 130 kDa region and between the Alzheimer's disease and young adult and elderly control groups in the 42 kDa region. There were no significant differences between the mean reflectance proportions of the three control groups in either the 130 or 42 kDa regions. The 110 kDa proportion was substantially higher and 65 kDa proportion substantially lower in the age-matched control group compared to the young adult control group.

**Identification of the 110, 65 and 42 kDa plasma APP bands as possible cleavage products of the 130 kDa APP species:**

When plasma APP from heparin-Sepharose eluates was incubated at 37 °C for 18 hours, the APP was slowly proteolytically degraded with a loss of the 130 kDa band and the accentuation of the three lower bands (110 kDa, 65 kDa and 42 kDa). Proteolysis was accelerated in the presence of  $Zn^{2+}$ . The zinc concentration required to stimulate proteolysis was 20  $\mu M$ , which is within the range of the normal human plasma concentration (Davies *et al*, 1968). The rate of  $Zn^{2+}$ -enhanced proteolysis was similar in Alzheimer's disease compared to young adult control samples (Figure 2), and identical degradation products were found in both groups. This indicated that the lower molecular weight APP forms in plasma could be degradation products of the 130 kDa form, and that proteolysis of the 130 kDa form in an Alzheimer's disease preparation changes the plasma APP profile towards that of control subjects.

$Zn^{2+}$ -enhanced APP proteolysis of a young adult control preparation over two hours was completely inhibited both by EDTA, heparin and the serine protease inhibitors aprotinin, diisopropyl fluorophosphate, SBTI and, incompletely inhibited by  $\alpha_1$ -ACT. Neither  $Al_3Cl$ , the cysteine-protease inhibitor N-ethyl maleimide, nor the acid-protease inhibitor pepstatin A influenced the reaction.

#### Investigations of the origin of plasma APP forms:

The APP signal detected by Western blotting was unaffected by ultracentrifugation of plasma (100,000g x 15 min). Plasma APP, as detected by MAb 22C11 on western blots of heparin-Sepharose eluates, could be immunoprecipitated by a rabbit antiserum raised against full-length native brain membrane-derived APP (90/3) and also by a rabbit antiserum raised against fusion protein APP<sub>695</sub> (anti-Fd-APP), but could not be immunoprecipitated by the preimmune serum of 90/3 or by rabbit antisera raised against synthetic peptides representing the carboxyl terminal 40 (anti-CT) or 100 (anti-A4CT) residues of APP. These data indicate that the APP forms observed in plasma are soluble and lack the carboxyl terminus.

20

To determine whether APP might be processed in whole plasma, fresh plasma from a young adult control was incubated at 37 °C over a period of eight days and the plasma APP assayed by heparin-Sepharose chromatography. No significant degradation of the 130 kDa APP form was observed over this time, indicating that constitutive processing of APP does not occur in plasma.

It has been previously reported that there is no difference between the content of APP in whole platelets or the electrophoretic pattern of platelet APP on Western blots in Alzheimer's disease cases compared to controls (Bush *et al*, 1990). Because other platelet abnormalities have been described in Alzheimer's disease (Zubenko *et al*, 1987), and also because APP is highly enriched in the platelet  $\alpha$ -granule (Bush *et al*, 1990; Bush *et al*, 1991 and Cole

*et al*, 1990), the possibility that platelet APP release contributes to the level of plasma APP was investigated. Platelets and plasma was examined from an individual with gray platelet syndrome (GPS), a congenital abnormality where there are greatly diminished contents in the platelet  $\alpha$ -granule. The GPS platelets contained <1% of the APP associated with normal platelets (Figure 3). In contrast, the 130 kd plasma APP purified by heparin-Sepharose chromatography was approximately 50% reduced in GPS compared to the control (Figure 3). These data indicate that the APP species seen in plasma are not likely to be an artefact caused by platelet release of APP during plasma preparation, but that platelet destruction, possibly in the spleen, may contribute a proportion of plasma APP.

### EXAMPLE 3

Studies of the zinc modulation of APP physiology.

- 15 I) Effect of zinc loading upon human plasma APP.
- 100 mg elemental zinc (as sulphate, in capsules) was given orally to two individuals with Alzheimer's disease (NINCDS/ADRDC criteria with abnormal plasma APP profiles) and two age-matched controls (with normal plasma APP profiles). Zinc was continued for 7 days and morning fasting bloods collected before the commencement of zinc supplements and three weeks after ceasing the supplement. Western blots with monoclonal antibody 22C11 were carried out on 65  $\mu$ g of plasma heparin-Sepharose eluate purified from the samples in the manner previously described. The results indicated an increase of 130 kDa species plasma APP relative to the other species present, in other words a shift towards the profile of APP species that is characteristic of Alzheimer's disease. The two control plasma APP profiles came to resemble Alzheimer's disease profiles, and the two Alzheimer's profiles worsened. The proportional increase in the 130 kDa species was approximately 20% per day of zinc supplementation. An elderly control volunteer who was given a four-fold higher dose over three days demonstrated the same changes in his plasma APP profile which persisted for three days after ceasing supplementation.

## II) Effect of lowering plasma zinc concentrations upon APP levels.

- Post-prandial plasma zinc levels are known to fall  $\approx 10\%$  in the hour after the meal. Seven Alzheimer's disease volunteers and six age-matched controls were
- 5 assayed for plasma APP profile and zinc levels both fasting and one hour after a standardized breakfast. Both plasma zinc and 130 kDa APP levels fell by  $\approx 10\%$  and there was a linear correlation between the change in APP level and the change in zinc concentration.
- 10 A young adult volunteer was given an oral dose of 50g glucose while fasting. Both plasma zinc and 130, 110 and 65 kDa APP levels fell by  $\approx 10\%$  within the first half hour after the glucose dose. Over a period of four hours with initial fall and subsequent rebound elevation in plasma zinc concentration was closely paralleled by plasma APP concentration as determined by APP
- 15 radioimmunoassay using an antibody raised against native brain APP (90/3).

## III) Measurement of plasma zinc concentrations in Alzheimer's disease.

Plasma zinc levels were shown not to be significantly elevated in fasting Alzheimer's disease plasma compared to age-matched controls.

20

## IV) Effect of zinc loading upon rat brain APP.

- 16 week old Sprague-Dawley male rats were injected daily for four days with 120mg/kg  $\text{ZnCl}_2$  solution. The animals were then anaesthetized, bled by intracardiac puncture and their brains removed, homogenized and separated
- 25 into membrane and soluble fractions following ultracentrifugation. Western blots of heparin-Sepharose eluates of plasma, membrane extracts and soluble fractions revealed the development of a novel 80 kDa band in plasma and a  $\approx 80\%$  increase in the amounts of all forms of APP in the zinc-supplemented rats compared to control plasma and brain preparations (taken from litter-
- 30 mates not receiving zinc).

V) Effect of extracellular zinc on cultured PC12 cells.

$10^6$  PC12 cells were plated for 24 hr in the presence of Dulbecco's modified Eagle's medium (DMEM, N2) supplemented with 8% fetal calf serum (FCS).

- 5 The medium was then removed and replaced with DMEM  $\pm$  2 to 50  $\mu$ M  $\text{ZnCl}_2$  or another salt, and no FCS. Cells and media were harvested 48 hr later and aliquots of media, cell cytosol and membrane (following lysis and ultracentrifugation) were assayed for APP by Western blotting with mAb 22C11. The results of four experiments with duplicated samples revealed that
- 10 the zinc induced a substantial increase in APP released into the media,  $\approx$ 50% at 2  $\mu$ M rising to a peak of  $\approx$ 200% at 50  $\mu$ M.  $\text{FeCl}_2$  had a similar effect and cuprous and aluminium chlorides had far smaller stimulatory effects. Smaller increases in cytosolic APP were seen accompanying these changes and there was no change in membrane associated APP levels.

15

VI) Effect of extracellular APP on zinc uptake by PC12 cells.

$10^6$  PC12 cells were plated for 24 hr in the presence of Dulbecco's modified Eagle's medium (DMEM, N2) supplemented with 8% fetal calf serum (FCS).

- The medium was then removed and replaced with DMEM with no FCS and
- 20 incubated for a further 24 hr. The cells were then washed (x2 with DMEM) and incubated with DMEM supplemented with 10 nM 130/110 kDa purified brain membrane-associated APP carrying 20 000 CPM  $^{65}\text{Zn}$  prepared as in Figure 1 above. Following varying periods of incubation, the cells were harvested, washed, exposed to Pronase digestion to remove surface proteins
- 25 and then lysed in scintillant and assayed in a beta-counter. The results indicated that  $\approx$ 10% of the counts were internalized in the presence of APP within an hour compared to  $\approx$ 4% in the absence of APP (two experiments with incubations performed in duplicate). These data indicate that APP may play a role in the cellular uptake of external  $\text{Zn}^{2+}$ .

30

## EXAMPLE 4

## BINDING OF ZINC TO APP

To determine whether APP binds zinc, incubations of human brain full-length APP with  $^{65}\text{Zn}^{2+}$  and competing concentrations of unlabelled  $\text{Zn}^{2+}$  were performed. Maximal binding was observed at 15 minutes (30%  $B_{\text{max}}$  at 1 minute) which was saturable at a dissociation constant ( $K_D$ ) of 764 nM at pH 7.4, and 2.08  $\mu\text{M}$  at pH 6.4. The binding of  $^{65}\text{Zn}^{2+}$  to APP was specific for all competing metal ions, including  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , with  $\text{Co}^{2+}$  the most competitive cation, able to compete off 70% of  $B_{\text{max}}$  at 20  $\mu\text{M}$ . Heavy metals,  $\text{Cu}^{2+}$  and  $\text{Al}^{3+}$  were able to compete off  $\approx 60\%$  of  $B_{\text{max}}$  at 20  $\mu\text{M}$ . The stoichiometry of  $\text{Zn}^{2+}$ -binding at APP was 1:1.

A putative  $\text{Zn}^{2+}$  binding site was identified by trypsin digestion of APP followed by amino-terminal sequencing of a 6 kDa digestion fragment which bound to  $\text{Zn}^{2+}$ -charged chelating-Sepharose. The fragment sequence was FRGVEFVXXPLA. To further characterise and confirm the  $\text{Zn}^{2+}$ -binding properties of this region of APP, candidate synthetic peptides were studied by dot blot. A synthetic peptide representing residues 181-214 of APP was able to bind  $\text{Zn}^{2+}$  in a saturable and specific manner. The role of the cysteine residues in contributing to this peptide's ability to bind  $^{65}\text{Zn}^{2+}$  was determined by studying the ability of the same peptide to bind  $^{65}\text{Zn}^{2+}$  where the cysteines in the peptide had been modified by carboxyamidomethylation, and by studying  $^{65}\text{Zn}^{2+}$  binding to another synthetic peptide representing residues 189-220 of APP<sub>695</sub>, lacking the cysteine residues at positions 186/187. Both of these peptides were able to bind  $^{65}\text{Zn}^{2+}$  significantly above background, but to only approximately 15% of the amount of  $^{65}\text{Zn}^{2+}$  binding that occurred using the same quantity of 181-214 peptide (or 181 to 200), indicating that the cysteine residues are obligatory for the zinc-binding properties of this peptide. Similar quantities of other peptides representing other regions of the APP molecule (residues 422-433, 581-601, 645-655) and other control peptides (renin and Insulin A chain) were unable to bind  $\text{Zn}^{2+}$ , whereas the positive control (Insulin B chain) bound  $^{65}\text{Zn}^{2+}$ .

To explore the functional significance of  $Zn^{2+}$  binding to APP, the effect of  $Zn^{2+}$  on heparin binding to APP derived from three sources; iodinated purified brain APP, partially purified unlabelled human brain APP, and partially purified unlabelled human serum APP, were studied. The amount of brain APP binding to heparin was increased  $\approx 50\%$  by the presence of  $50\mu M$   $Zn^{2+}$  specifically. The increase in  $^{125}I$ -APP binding to heparin reached a plateau at  $75\mu M$   $Zn^{2+}$ .  $Zn^{2+}$  increased the proportion of higher-affinity binding of APP to heparin, increasing the amount of  $^{125}I$ -APP that was recovered from the 50-2000 mM NaCl elution fraction by approximately 3-fold in the presence of  $50\mu M$   $Zn^{2+}$  and by 4.5-fold in the presence of  $100\mu M$   $Zn^{2+}$ .  $Ca^{2+}$  and  $Mg^{2+}$  did not alter the profile of  $^{125}I$ -APP elution,  $Co^{2+}$  at  $75\mu M$  increased the recovery of  $^{125}I$ -APP in the 100-500 and 500-2000 mM NaCl fractions, and  $Al^{3+}$  decreased the recovery of  $^{125}I$ -APP except in the 500-2000 mM NaCl fraction where recovery was increased approximately 4.5-fold.

$Zn^{2+}$  at  $50\mu M$  had a similar effect on the NaCl gradient elution profile of partially purified brain APP bound to heparin-Sepharose. The presence of  $Zn^{2+}$  caused an increase in the amount of APP recovered in all fractions from 1110 to 1644 reflectance units, an increase of 48%, and also caused an increase in APP recovery in fractions eluted by NaCl concentrations above 520 mM.

Despite the increase in APP recovery in the eluted fractions, protein assay showed a 13% reduction in the amount of eluted protein, indicating that the presence of  $Zn^{2+}$  increased the specificity of APP binding to heparin.  $^{125}I$ -APP recovery occurred over a range of NaCl concentrations 200-300 mM lower than partially purified brain APP elution. This may be the result of radiolytic or oxidative damage to the APP molecule during iodination, although the possibility that the elution of partially purified APP is influenced by co-purified material cannot yet be excluded.



The presence of 50  $\mu\text{M}$   $\text{Zn}^{2+}$  promoted a smaller increase in the binding of partially purified serum APP to heparin-Sepharose. In serum samples, the increase in APP recovered from the NaCl elution was  $\approx 10\%$ , however protein assay of recovered fractions indicated that, unlike the effect of  $\text{Zn}^{2+}$  upon heparin-Sepharose chromatography of partially purified brain proteins, the effect of  $\text{Zn}^{2+}$  upon the heparin-Sepharose chromatography of partially purified serum was to increase the amount of protein recovered by 12%. Unlike its effect on the brain-derived APP salt elution profile, the presence of  $\text{Zn}^{2+}$  in the heparin-Sepharose chromatography of serum-derived APP did not increase the proportion of higher-affinity binding.

The effect of  $\text{Zn}^{2+}$  in modulating heparin binding to APP was also studied in a Biosensor system. At 100  $\mu\text{M}$ ,  $\text{Zn}^{2+}$  was shown to strongly promote heparin binding to APP purified from rat brain. This effect was most pronounced and most specific for  $\text{Zn}^{2+}$ , as opposed to the absence of divalent cations or the presence of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  or  $\text{Co}^{2+}$ , and was most evident when the heparin:APP ratio was low. Concentrations of  $\text{Zn}^{2+}$  as low as 50 nM had a marked effect on increasing heparin binding to rat brain APP.  $\text{Zn}^{2+}$  at 50 nM promoted a  $\approx 170\%$  increase in heparin binding, the effect saturating at 70  $\mu\text{M}$ .

#### EXAMPLE 5

##### HEPARIN PROTECTS APP FROM PROTEOLYTIC CLEAVAGE

##### ZINC ABOLISHES THE PROTECTIVE EFFECT OF HEPARIN

The proteolytic activity of trypsin (Boehringer, Mannheim) was studied to determine whether it was modulated by a range of doses of heparin (Sigma) and  $\text{ZnCl}_2$ . Both heparin and  $\text{ZnCl}_2$  left tryptic activity unaltered as measured by its ability to cleave a fluorogenic synthetic substance Z-F-R-AMC, indicating that trypsin is a suitable serine protease to employ in studies of the modulation of APP proteolytic resistivity by heparin and zinc. Digestions of human brain membrane-derived APP (which contains the intact carboxyl

terminus) were carried out with trypsin in an amount ratio of 1:64 (enzyme:substrate) for one hour at 37 °C. Western blots for APP using the APP binding monoclonal antibody 22C11 were used to monitor the progression of the proteolytic reaction and to detect breakdown products. It was found  
5 that the presence of heparin in concentrations as low as 100 nM caused a marked reduction in the rate of degree of brain APP degradation by trypsin. This protective effect saturated at 10 µM.

The presence of  $Zn^{2+}$  (up to 100 µM) or EDTA (1 mM) had no effect on the  
10 rate of APP proteolysis by trypsin in this reaction. However, the presence of  $Zn^{2+}$  (above 1 µM) with heparin (1 µM) in the proteolytic reaction completely abolished the protective effect of heparin. This was an unexpected response given that previous results showed that the presence of  $Zn^{2+}$  promotes heparin binding to APP.

15

One possible explanation for this finding is that zinc binding to APP could promote heparin binding at a different site on the protein, known to be towards the center of APP, by enhancing the protein's conformational stability. The conformational stabilisation caused by zinc may promote the central  
20 region of APP to remain open to heparin attachment to residues 98 to 105 of APP<sub>695</sub> (CKRGRKQCKTH) or residues 318 to 331 of APP<sub>695</sub> (KAKERLEAKHRER), perhaps by a charge effect. When heparin binding occurs in the absence of zinc, the protein may be induced to assume a more globular and protease-resistant conformation. This zinc binding to APP could  
25 stabilise the APP to the extent that heparin binding is prevented from inducing the alteration in APP conformation which increases protease resistivity.

### EXAMPLE 6

#### ADMINISTRATION OF ZINC IN ALZHEIMER'S DISEASE (AD)

30 The subjects from Example 3 were studied.

The healthy volunteers suffered no ill effects from the zinc supplementation.

The two AD volunteers became acutely unwell while on zinc supplementation.

They both suffered a severe loss of cognitive function with mini-mental state examination (Folstein et al., 1975) scores deteriorating from moderately demented levels to unrecordable. Eye movement abnormalities and general levels of self care worsened over the period of supplementation. This response  
5 was consistent with a neurotoxic response to the zinc supplementation. When zinc supplementation was ceased, cognitive function returned to the previous levels within two weeks.

Accordingly, a diagnostic test may involve an oral zinc challenge. A clinically  
10 measured neurotoxic response would be compatible with a diagnosis of AD.

The interaction between APP, zinc and heparin is currently not well understood, although from the description herein it is clear that both zinc and heparin interact with APP through specific binding sites modulating its stability  
15 to proteolysis. The inventors have identified a zinc binding site and a heparin binding site on APP. There may be additional zinc and heparin binding sites on the APP molecule which modulate its stability. This invention extends to modulating the interaction of zinc and/or heparin and/or other agents with APP to treat, ameliorate or prevent Alzheimer's disease and other neurological  
20 disorders associated with aberrant processing of APP.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations  
25 and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Table 1. Ratios of plasma APP forms analysed by image capture in Alzheimer's disease and controls.

- 5 Heparin-Sepharose eluates of Alzheimer's disease and control plasma samples were immunoblotted with MAb 22C11 and the reflectances of the bands at 130, 110, 65 and 42 kDa were measured by computer-assisted image capture analysis (see Example 1). The relative amounts of the four APP derivatives, as percentages of total lane signal, were determined in each plasma sample and
- 10 averaged to give the values presented here. Independent samples t-tests between pooled controls and Alzheimer's disease groups were performed in the four regions at a significance level of 0.0125. Comparisons were significant for only two regions, 130 and 42 kDa (two-tailed,  $p < 0.001$ ). Comparisons
- 15 between all pairs of diagnostic groups (Alzheimer's disease, other neurological disease controls, normal young adult controls and non-demented age-matched controls) were then performed for the 130 and 42 kDa bands (see Example 1).

TABLE 1

	Mean age $\pm$ SD	Percentage of Total APP $\pm$ SD			
		130 kDa	110 kDa	65 kDa	42 kDa
AD, n=27	64.8 $\pm$ 6.4	37.5 $\pm$ 11.0	23.2 $\pm$ 5.7	21.9 $\pm$ 8.0	17.3 $\pm$ 6.8
Pooled controls, n=34	52.2 $\pm$ 16.7	22.8 $\pm$ 6.3 ***	24.7 $\pm$ 7.1 NS	26.4 $\pm$ 7.4 NS	26.5 $\pm$ 8.9 ***

Neurological controls, n=6	64.0 $\pm$ 6.8	25.0 $\pm$ 8.3 *	22.7 $\pm$ 5.8	31.0 $\pm$ 3.2	21.3 $\pm$ 10.6 NS
Age-matched controls, n=15	64.5 $\pm$ 3.8	20.1 $\pm$ 5.1	30.1 $\pm$ 5.0	21.0 $\pm$ 4.0	28.8 $\pm$ 7.2 *
Young adult controls, n=13	32.5 $\pm$ 6.9	24.8 $\pm$ 5.9	18.5 $\pm$ 4.1	30.5 $\pm$ 7.9	26.2 $\pm$ 9.3

\*\*\* t-test,  $p < 0.001$ \* Scheffe,  $p < 0.05$ 

NS No significance

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## CLAIMS:

1. A method of assaying for Alzheimer's disease in a human said method comprising isolating a sample of circulatory fluid from said human, determining the amount of the 130 kDa form and/or 42 kDa form of amyloid precursor protein (APP) and/or any derivatives of either form of APP in said fluid relative to a normal control wherein a relative increase in the 130 kDa form and/or its derivative and/or a relative decrease in the 42 kDa form and/or its derivative is indicative of the disease.
2. The method according to claim 1 wherein the circulatory fluid is obtained after the human has undergone at least four hours of fasting.
3. The method according to claim 1 wherein the circulatory fluid is blood plasma.
4. The method according to any one of claim 1 to 3 wherein the amounts of the 130 kDa and/or 42 kDa forms of APP and/or their derivatives are determined by Western blot.
5. A method of assaying for Alzheimer's disease in a human said method comprising isolating a sample of genetic material from said human, contacting said genetic material to an oligonucleotide capable of hybridising within or near the gene encoding APPase and ascertaining whether or not said APPase gene contains any nucleotide aberrations relative to the naturally occurring gene.
6. The method according to claim 5 wherein the oligonucleotide is a probe and aberrations in the gene are determined by the extent of hybridisation to a region within said gene.

7. The method according to claim 5 wherein the oligonucleotide is a primer capable of directing amplification of all or part of the APPase gene and aberrations are detected by sequencing amplification products or measuring the profile of amplification products relative to a normal control.
8. A method for assaying for Alzheimer's disease in a human said method comprising isolating a sample of circulatory fluid from said human and contacting said fluid to a binding effective amount of an antibody specific to APPase and determining the amount of APPase in said fluid.
9. The method according to claim 8 wherein the circulatory fluid is blood pressure.
10. The method according to claim 8 wherein the antibody is a monoclonal antibody.
11. The method according to claim 8 wherein binding of the antibody is determined by contacting the sample with a second antibody labelled with a reporter molecule and assaying a signed product by said reporter molecule.
12. The method according to claim 8 or 10 wherein the antibody is specific only for naturally occurring, active APPase.
13. A kit for assaying for Alzheimer's disease in a human, said kit comprising in compartmental form a first compartment adapted to contain an antibody specific to the 130 kDa form and/or 42 kDa form and/or derivatives thereof and/or to APPase; optionally a second compartment adapted to contain a second antibody specific to said first antibody and labelled with a reporter molecule.

14. The kit according to claim 13 further comprising means for conducting a Western blot.

15. A method for treating Alzheimer's disease in a patient comprising subjecting said patient to a means for modulating divalent or trivalent cation and/or heparin interaction with APP.

16. The method according to claim 15 wherein the cation is a divalent cation and is zinc.

17. The method according to claim 16 wherein a therapeutically effective amount of a zinc binding agent is administered to said patient, which agent is capable of binding zinc and thereby modulating its interaction with APP.

18. The method according to claim 17 wherein the zinc binding agent is one or more of phytic acid and its derivatives, desferrioxamine, sodium citrate, EDTA, 1,2-diethyl-3-hydroxypyridin-4-one and 1-hydroxyethyl-3-hydroxy-2-methylpyridin-4-one.

19. The method according to claim 17 wherein the zinc binding agent is orally administered.

20. The method according to claim 16 wherein said patient is subjected to a diet low in free zinc.

21. The method according to claim 16 wherein an agent is administered to said patient which is capable of blocking one or more components of a zinc transport system so as to reduce zinc uptake.

22. A method of detecting Alzheimer's disease in a patient which method comprises administering to said patient a challenge of zinc and thereafter assessing neurological function wherein a decrease in neurological function compared relative to normal controls is indicative of Alzheimer's disease.
23. The method according to claim 22 wherein administration of the zinc challenge is by oral, intravenous, intramuscular, transdermal, rectal or intranasal administration.
24. The method according to claim 22 wherein administration is oral.

Figure 1A

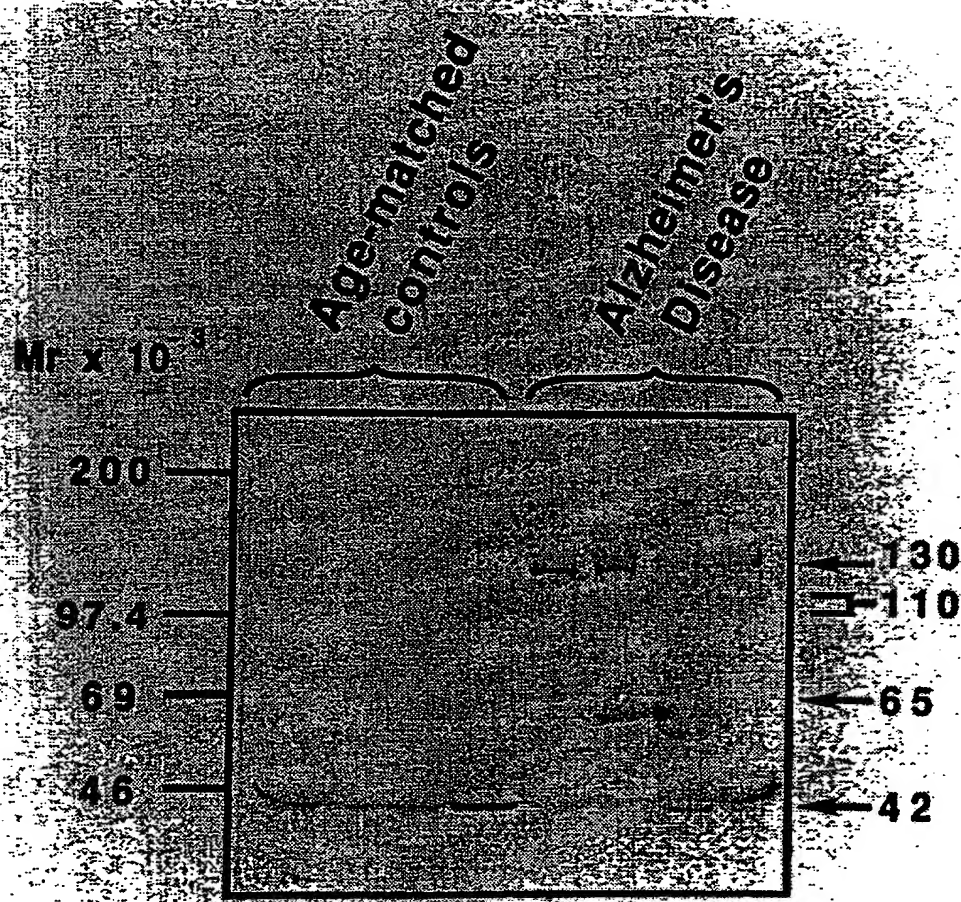


Figure 1B

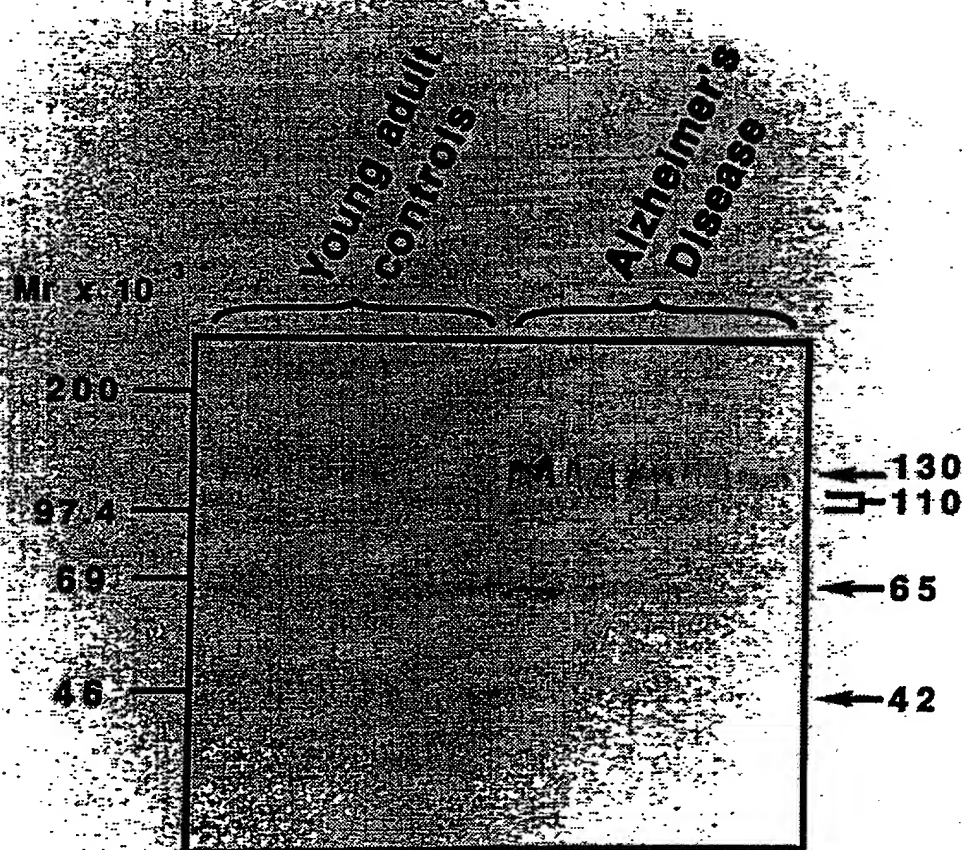
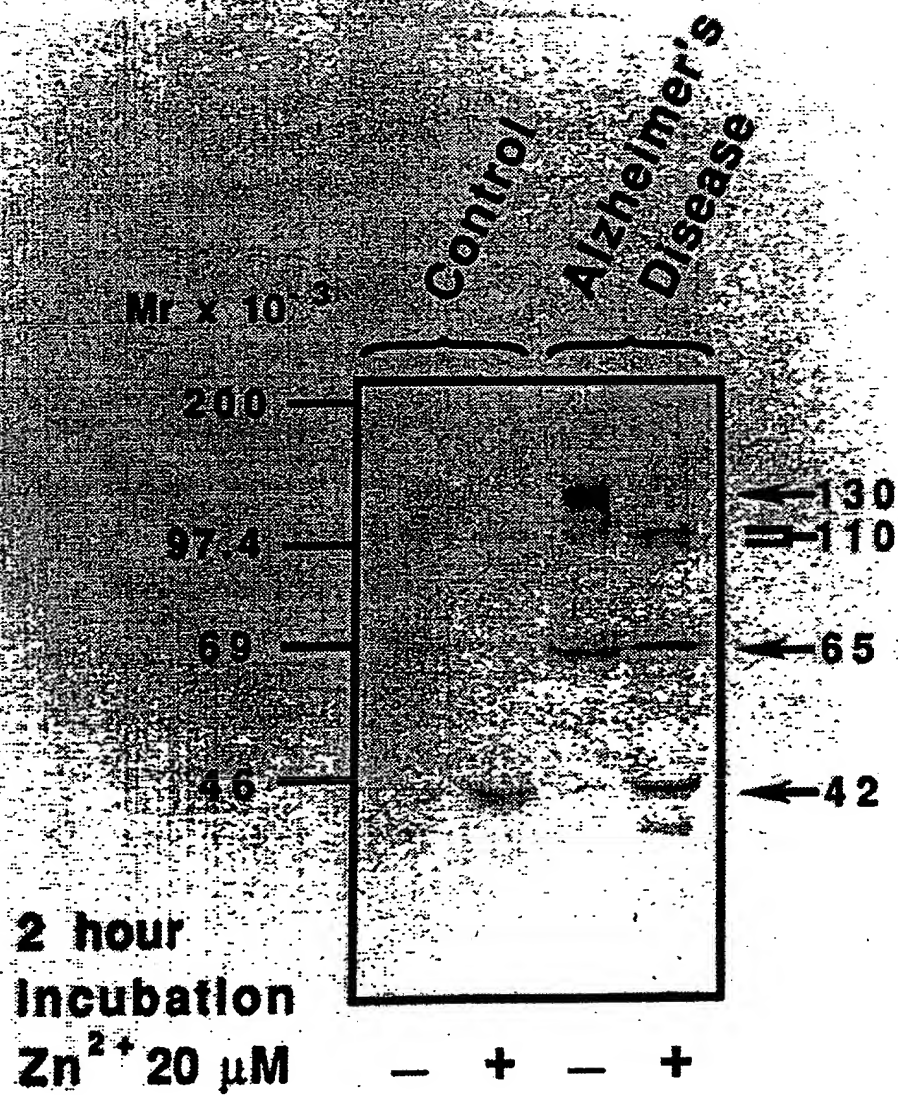
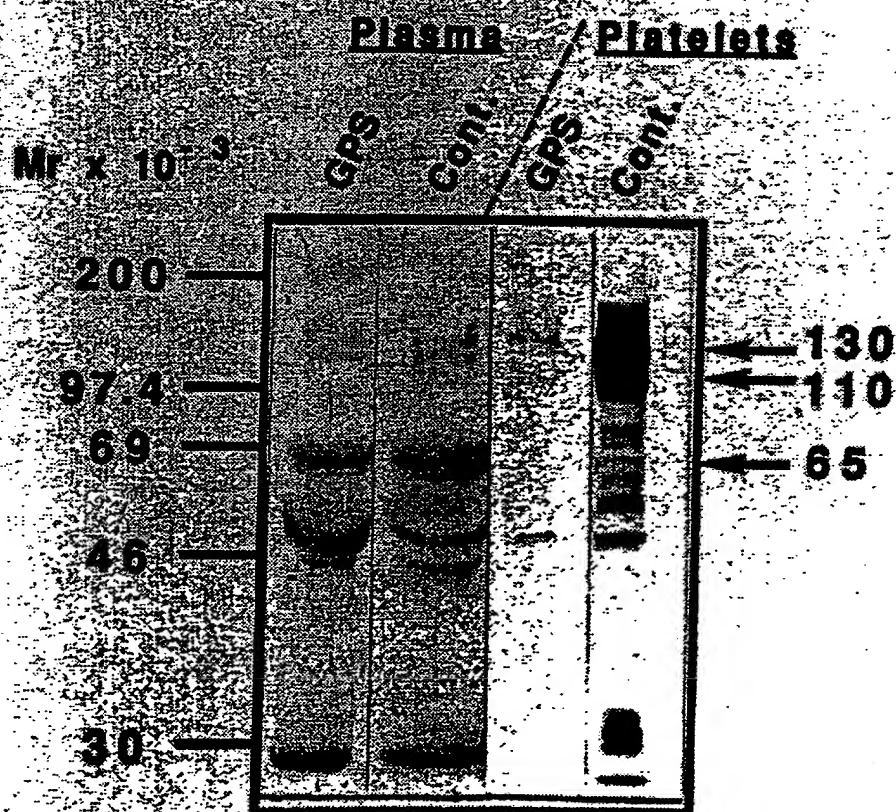


Figure 2

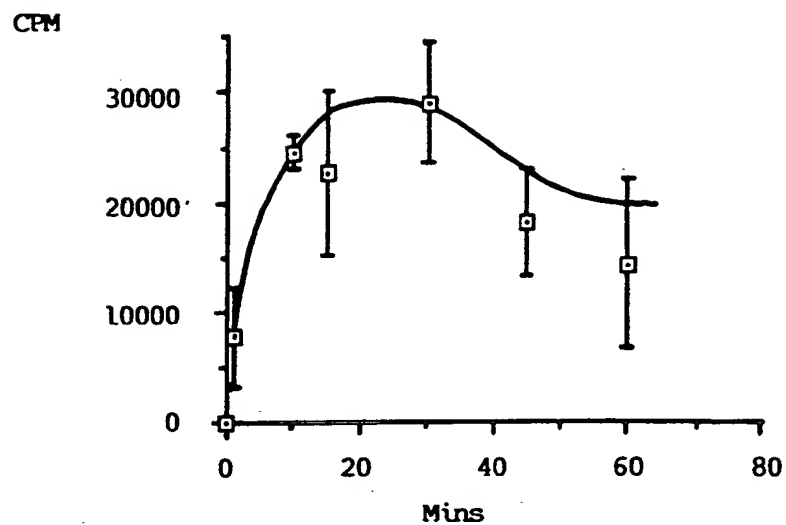
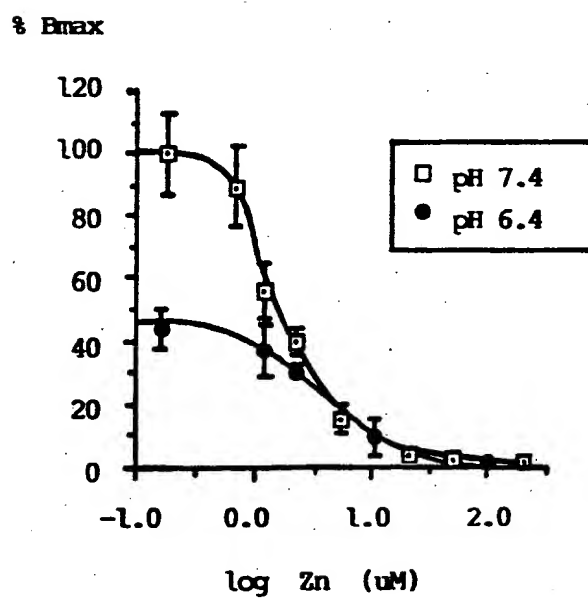




**FIG 3**

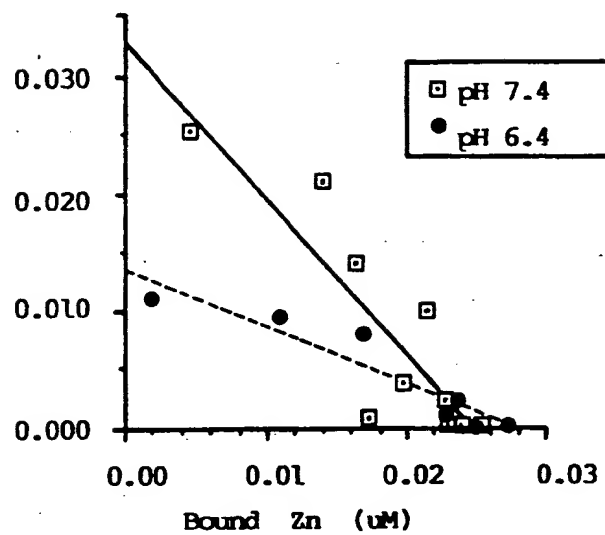


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FIGURE 4FIGURE 5

6/10

Bound Zn /free

FIGURE 6

7/10

% Bmax (APP and Zn65 alone)

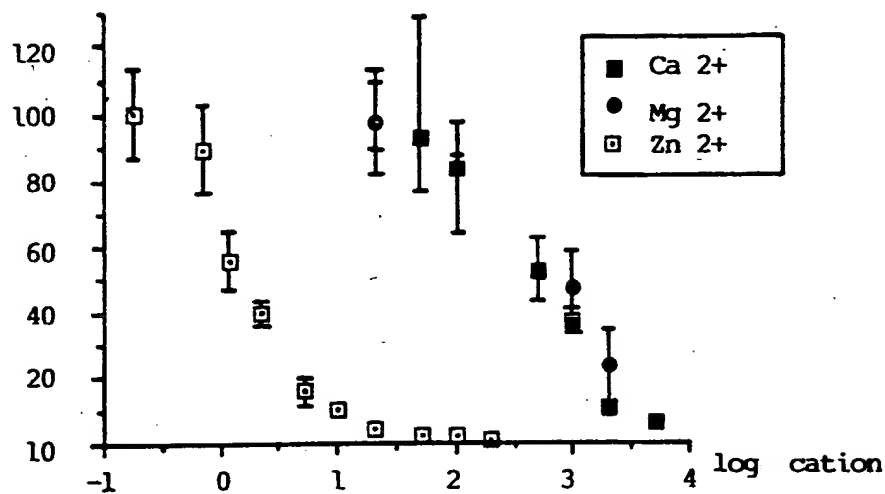


FIGURE 7 (a)

% Bmax (Zn65 and APP alone)

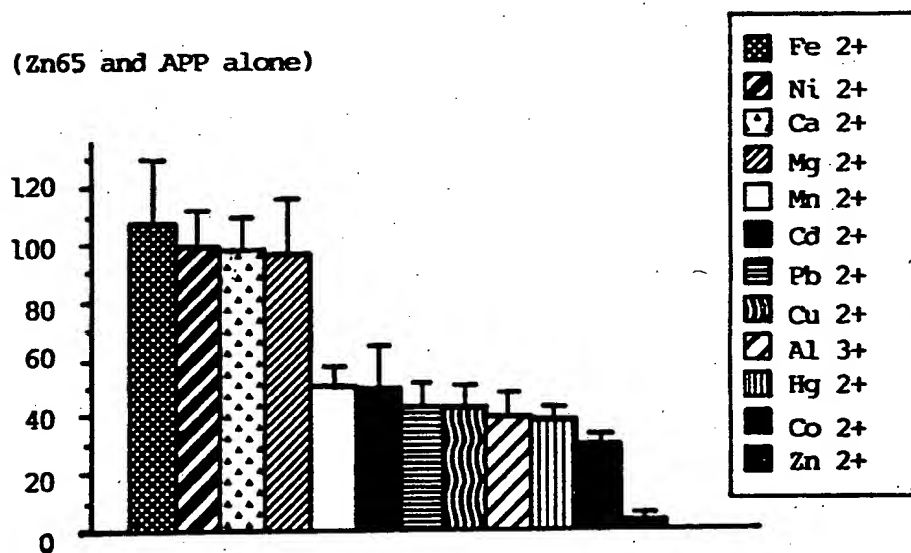
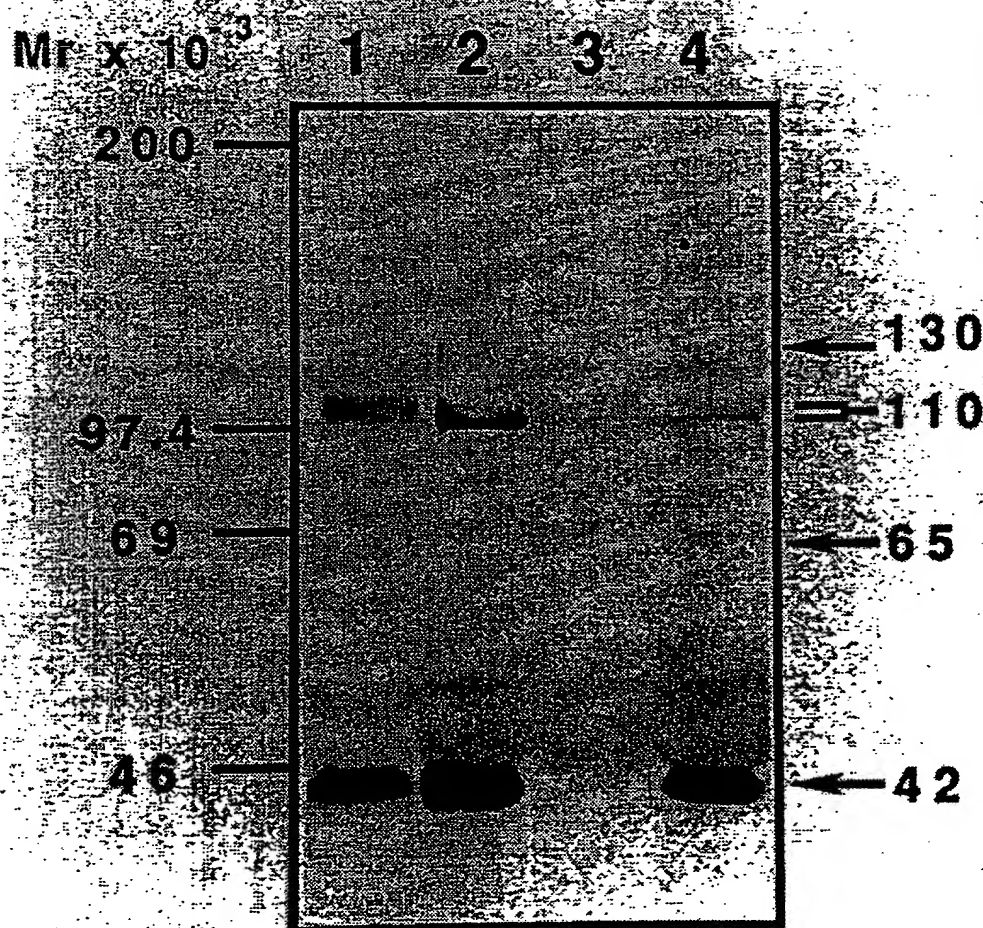
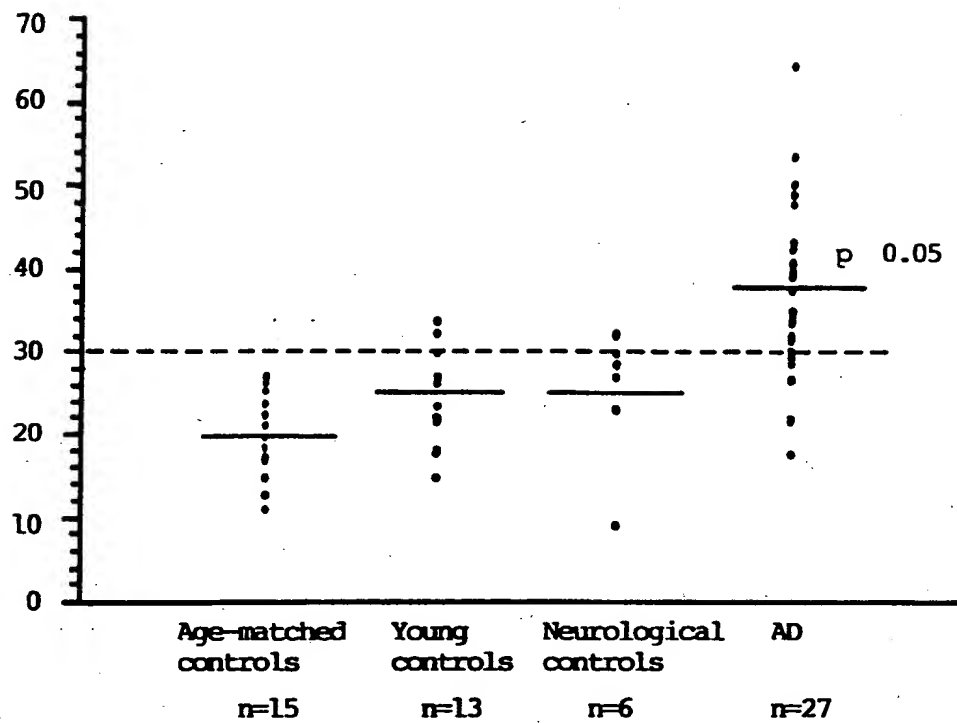


FIGURE 7 (b)

FIG 8

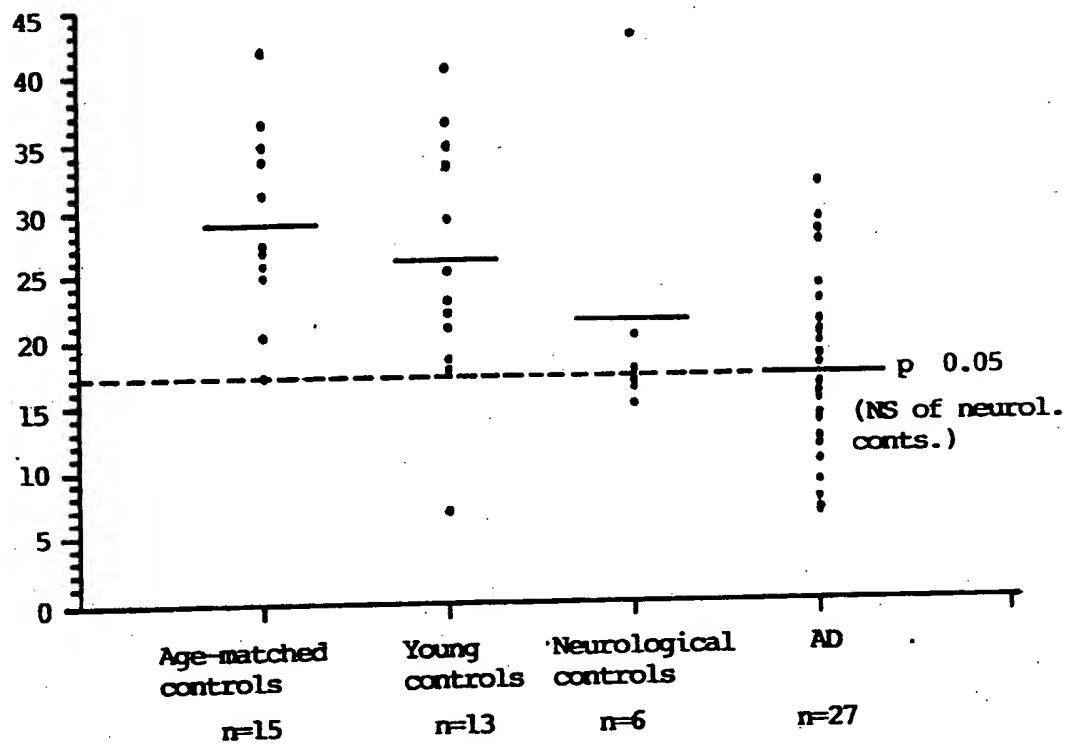
9/10

% APP immunoreactivity


FIGURE 9(a)

10/10

% APP immunoreactivity

FIGURE 9 (b)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
X	WO,A,91/16629 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) published 31 October 1991 (31.10.91) see examples 1-22 in particular	1-4,13
X	SELKOE, D.J. et al. (1988) $\beta$ -amyloid precursor protein of Alzheimer disease occurs as 110- to 135- kilodalton membrane associated proteins in neural and non-neural tissues, Proc. Natl. Acad. Sci. USA, vol. 85, pp 7341-7345, published October 1988	1,13
P,X	Derwent WPAT online abstract accession No. 92-346529, JP,A,04252956 (ASAHI CHEM. IND. CO. LTD) 8 September 1992 (08.09.92)	1,13
P,X	Derwent WPAT online abstract accession No. 92-346528, JP,A,04252955 (ASAHI CHEM. IND. CO. LTD) 8 September 1992 (08.09.92)	1,13
P,X	Derwent WPAT online abstract accession no. 92-346527, JP,A,04252954 (ASAHI CHEM. IND. CO. LTD) 8 September 1992 (08.09.92)	1,13
P,X	Derwent WPAT online abstract accession No. 92-189677, JP,A,04126095 (ASAHI CHEM. IND. CO. LTD) 27 April 1992 (27.04.92)	1,13
A	AU,A,33365/89 (BIONIX CORPORATION) published 2 November 1989 (02.11.89)	15-24

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> Int. Cl. <sup>5</sup> G01N 33/564, 33/577, C12Q 1/68, A61K 33/30, 31/66, A61K 31/16, 31/19, 31/44 According to International Patent Classification (IPC) or to both national classification and IPC				
<b>B. FIELDS SEARCHED</b>  Minimum documentation searched (classification system followed by classification symbols) IPC <sup>5</sup> G01N 33/564, 33/577, C12Q 1/68  Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched CHEMICAL ABSTRACTS BIOTECH ABSTRACTS  Electronic data base consulted during the international search (name of data base, and where practicable, search terms used) DERWENT DATA BASES; FILE WPAT; KEYWORDS: ALZHEIMERS AND ZINC, ALZHEIMERS AND AMYLOID PRECURSOR PROTEIN, APP; CHEM. ABS.; KEYWORDS: ALZHEIMERS, AMYLOID PRECURSOR PROTEIN, ANTIBODY AND OLIGONUCLEOTIDE AND PROBE; BIOTECH. ABS.; KEYWORDS: ALZHEIMERS AND AMYLOID PRECURSOR PROTEIN				
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>				
<b>Category*</b>	<b>Citation of document, with indication, where appropriate, of the relevant passages</b>	<b>Relevant to Claim No.</b>		
P,X	AU,A,82153/91 (CASE WESTERN RESERVE UNIVERSITY) earliest priority date 29 June 1990 (20.06.90) see page 6, line 17 - page 9, line 10 in particular	1,13		
P,X	AU,A,11694/92 (IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY AND MEDICINE) earliest priority date 21 January 1991 921.01.91) see claims 27-28 in particular	1,13		
X	AU,A,58173/90 (E.I. DU PONT DE NEMOURS AND COMPANY) published 7 January 1991 (07.01.91) see whole document	1,13		
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.         </div> <div style="width: 45%;"> <input checked="" type="checkbox"/> See patent family annex.         </div> </div>				
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top;"> <p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </td> <td style="width: 50%; vertical-align: top;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p> </td> </tr> </table>			<p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>
<p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>			
Date of the actual completion of the international search 18 February 1993 (18.02.93)		Date of mailing of the international search report 25 FEBRUARY 1993 (25.02.93)		
Name and mailing address of the ISA/AU.  AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA  Facsimile No. 06 2853929		Authorized officer  <div style="text-align: center;">           JOHN ASHMAN          Telephone No. (06) 2832364       </div>		



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Patent Document Cited in Search Report		Patent Family Member			
AU	82153/91	WO	9200521		
AU	11694/92	GB	9118445	WO	9213069 GB 9101307
AU	58173/90	CA	2017832	EP	474765 JP 4505865
		WO	9015331		
WO	9116628				
JP	04252956				
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